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## HUMANIZATION OF A MOUSE ANTI-HUMAN INTERLEUKIN-6 RECEPTOR ANTIBODY COMPARING TWO METHODS FOR SELECTING HUMAN FRAMEWORK REGIONS\*

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**Abstract**—Mouse monoclonal antibody AUK12-20 binds to human IL-6 receptor and inhibits IL-6 functions. It has been humanized by CDR-grafting for therapeutic use. In the design of reshaped human AUK12-20 V<sub>L</sub> region, the human framework regions (FRs) from the human Bence-Jones protein REI were used. The reshaped human AUK12-20 light chain, in combination with chimeric AUK12-20 heavy chain, bound to antigen as well as chimeric AUK12-20 antibody. In the design of reshaped human AUK12-20 V<sub>H</sub> region, two sets of the human FRs were chosen and compared. One set was from the consensus amino acid sequence for human V<sub>H</sub> regions subgroup (HSG)-I and the other set was from human antibody HAX, the most similar human V<sub>H</sub> region found in a database of human immunoglobulin sequences. The HSG-I-based and the HAX-based reshaped human AUK12-20 heavy chains in combination with the reshaped human AUK12-20 light chain, showed approximately 90 and 100% antigen-binding and competition-binding activities as compared to the chimeric or mouse AUK12-20 heavy chains. Most importantly, these humanized antibodies inhibited the IL-6-dependent tumor cell growth as well as the original mouse antibody suggesting that these humanized antibodies could be efficacious in human patients. Our results show that both approaches for the design of reshaped human antibodies can be used for successful humanization. The approach based on FRs from the most similar individual human antibody, however, seemed to be best for designing a reshaped human antibody that mimicked as closely as possible the original mouse antibody.

**Key words:** humanization, monoclonal antibody, IL-6 receptor, anti-cancer.

### INTRODUCTION

A large number of mouse monoclonal antibodies (Mabs) have been proposed for use as therapeutic agents against cancer, infectious disease and thrombosis (Larrick, 1989; Grossbard *et al.*, 1992). Mouse Mabs, however, are highly immunogenic in humans and, for this reason, their therapeutic value in humans is limited. The half-life of mouse Mabs in humans is very short (approximately 15 hr) (Khazaeli *et al.*, 1988). The mouse Mabs cannot

be administered in multiple doses without generating an immune response which not only interferes with the planned efficacy but also risks an adverse allergic response in the patient. One solution is to genetically-engineer the mouse Mabs to look like human antibodies.

Mouse Mabs can be humanized in two ways. The more simple method is to construct chimeric antibodies in which the variable regions from the mouse Mabs are linked to human constant regions. Chimeric antibodies have a substantial reduction in the percent of the amino acid sequence derived from a non-human source and, therefore, are expected to be less immunogenic than the original mouse antibody. However, an immune response to the mouse variable regions can still occur (LoBuglio *et al.*, 1989; Khazaeli *et al.*, 1991; Saleh *et al.*, 1992).

The second method for humanizing Mabs is more complicated but more extensively reduces their potential immunogenicity in humans. In this method, the complementarity determining regions (CDRs) from the variable regions of the mouse Mab are grafted into human variable regions to create "reshaped" human variable regions (Riechmann *et al.*, 1988; Verhoeven *et al.*, 1988).

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**Abbreviations:** IL-6, interleukin-6; Mab, monoclonal antibody; CDR, complementarity determining region; FRs, framework regions; V<sub>H</sub>, heavy chain variable region; V<sub>L</sub>, light chain variable region; HSG, human subgroup; PCR, polymerase chain reaction.

The only portions of the final reshaped human antibody derived from non-human protein sequence are the CDRs. CDRs are highly variable amino acid sequences. A reshaped human antibody carrying mouse CDRs should not be any more immunogenic than a natural human antibody containing human CDRs. The binding affinities of such reshaped human antibodies depend heavily on the human FRs used. Although some improvement can be achieved by changing amino acid residues in the human FRs, minimal changes should be made in order to avoid introducing immunogenic sites. To achieve this, first, the human FRs must be carefully selected, and second, potentially important residues in the FRs must be identified.

Two previous publications have reported testing human FRs selected from two different human sequences. Human FRs from the V<sub>H</sub> region of human NEW antibody and from the consensus sequence for human V<sub>H</sub> region belonging to subgroup I (HSG-I) (Kabat *et al.*, 1991) were compared in the humanization of mouse 0.5 $\beta$  V<sub>H</sub> region (Maeda *et al.*, 1991). Although several alterations in the FRs were tested, only the HSG-I-based reshaped human 0.5 $\beta$  V<sub>H</sub> regions showed good antigen-binding. Human FRs from the V<sub>H</sub> regions of KOL and NEW antibodies were compared in the humanization of rat CAMPATH-9 V<sub>H</sub> region (Gorman *et al.*, 1991). Only the KOL-based reshaped human heavy chains showed good antigen-binding. The success of the HSG-I-based 0.5 $\beta$  V<sub>H</sub> regions and the KOL-based CAMPATH-9 V<sub>H</sub> regions were probably due to the higher degree of homology between the V<sub>H</sub> regions of rodent antibody and human antibody used as a recipient for CDR-grafting. The V<sub>H</sub> regions of mouse 0.5 $\beta$  and rat CAMPATH-9 antibodies are most similar to the consensus sequence for HSG-I and HSG-III, respectively. KOL is a member of HSG-III and NEW is a member of HSG-II. These reports indicate that successful humanization depends on selecting human FRs from the most homologous HSG to the V<sub>H</sub> region of rodent antibody. More recently, human FRs from the V<sub>H</sub> regions of GAL, JON and NEW antibodies were also compared in the humanization of mouse 1B4 V<sub>H</sub> region (Singer *et al.*, 1993). The V<sub>H</sub> region of mouse 1B4 antibody is most similar to the consensus sequence for HSG-III. Although both the V<sub>H</sub> regions of GAL and JON antibodies belong to HSG-III, the V<sub>H</sub> region of GAL antibody gave much better antigen-binding than that of the JON antibody when no alterations were made in the FRs. This was probably due to the higher degree of homology between the FRs of mouse 1B4 and GAL (84% identity) as compared to mouse 1B4 and JON (81% identity). Three alterations in the FRs of the GAL-based reshaped human 1B4 V<sub>H</sub> region were required to achieve antigen-binding activity that was equivalent to the parent mouse 1B4 V<sub>H</sub> region.

We have previously reported successful humanization of mouse PM-1 antibody which is specific for the human interleukin-6 receptor (IL-6) (Sato *et al.*, 1993). PM-1 antibody suppresses strongly IL-6-dependent tumor cell growth both *in vitro* and *in vivo* (Sato *et al.*, 1993; Suzuki

*et al.*, 1992; Okuno *et al.*, 1992). Mouse Mab AUK12-20 antibody also binds to human IL-6R and inhibits IL-6 functions but AUK12-20 antibody recognizes a different epitope from PM-1 antibody (manuscript in preparation). This report describes the design and construction of reshaped human AUK12-20 antibodies. In particular, we have compared two approaches to the design of reshaped AUK12-20 V<sub>H</sub> regions. We have based the design either on the most homologous consensus sequence or on the most homologous human antibody. Both approaches have resulted in successful reshaped human AUK12-20 antibodies. The approach based on using the most homologous antibody, however, seemed to be best at giving a reshaped human AUK12-20 antibody that mimicked as closely as possible the original mouse antibody.

## MATERIALS AND METHODS

### *cDNA cloning*

Total RNA was prepared from the hybridoma cells according to a standard guanidinium cesium chloride method. First-strand cDNA synthesis was achieved using 5  $\mu$ g of total RNA and the cDNAs encoding V<sub>L</sub> and V<sub>H</sub> regions were amplified using PCR primers designed for rapidly cloning entire mouse antibody variable regions (Jones and Bendig, 1991). After cloning the cDNAs into pUC19 vectors, the DNA sequences were determined. The amino acid sequences of the V<sub>L</sub> and V<sub>H</sub> regions were compared, first to the sequences of all known mouse and human antibody variable regions as found in Leeds (OWL Composite Protein Sequence Database, University of Leeds, U.K.), and second to the sequences of immunoglobulin variable regions present in the Brookhaven database of protein structures.

### *Molecular modeling of mouse AUK12-20 variable regions*

A model was built on a Silicon Graphics IRIS 4D workstation running under the UNIX operating system and using the molecular modeling package QUANTA (Polygen Corp., U.S.A.). The V<sub>L</sub> region was modeled on the solved structure of mouse MCPC603 antibody (Brookhaven code 1MCP). The V<sub>H</sub> region was modeled on the structure of the mouse R19.9 antibody (Brookhaven code 1F19). The V<sub>L</sub> and V<sub>H</sub> regions of mouse AUK12-20 antibody have 66 and 68% identity, respectively, to mouse MCPC603 and R19.9 antibodies. Identical residues in the FRs were retained and non-identical residues were substituted using QUANTA. CDR2 (L2) and CDR3 (L3) of the V<sub>L</sub> region and CDR1 (H1) and CDR2 (H2) of the V<sub>H</sub> region from mouse AUK12-20 antibody corresponded to canonical structures postulated by Chothia *et al.* (1987, 1989). Since there were no canonical structures for CDR1 (L1) of the V<sub>L</sub> region and CDR3 (H3) of the V<sub>H</sub> region, the Brookhaven databank of protein structures was searched for possible loop structures on which to base the modeling of L1 and H3. For L1, the loop was modeled a loop (residues 45 onwards) from myoglobin protein (Brookhaven code 1MBO). For H3, the loop was

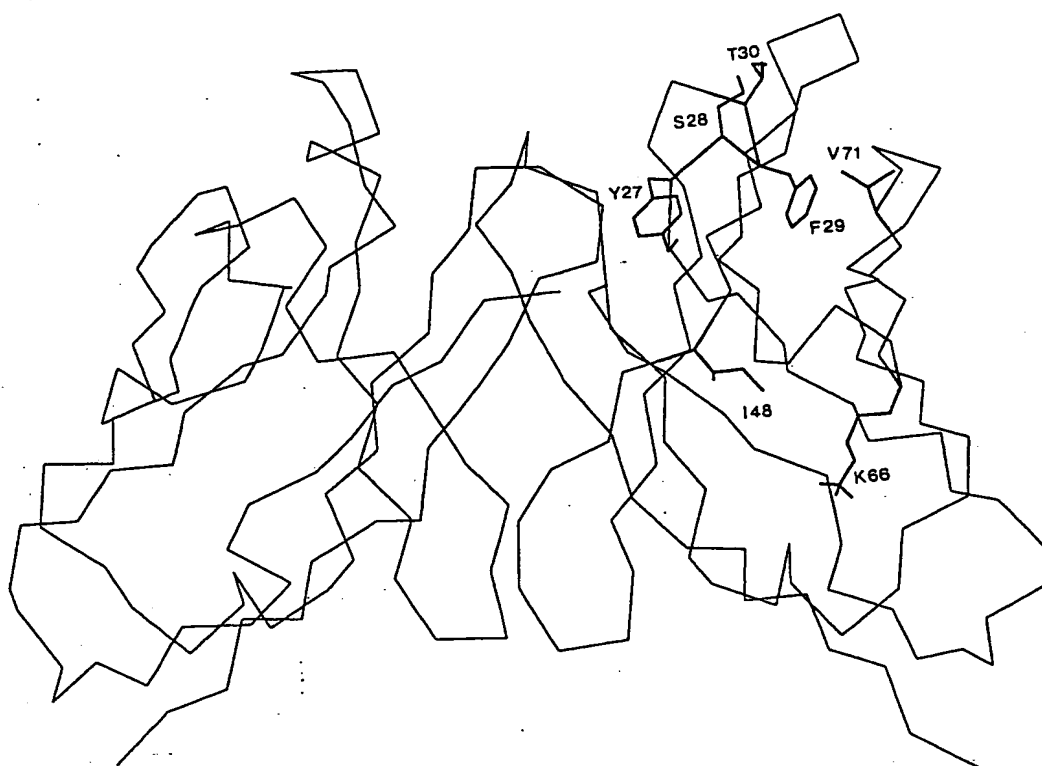


Fig. 1. An  $\alpha$ -carbon trace of the variable regions of the mouse AUK12-20 antibody model. Framework side-chains of interest in this work are shown by bold lines.

modeled a loop (residue 235 onwards) from bacteriochlorophyll A protein (Brookhaven code 3BCL). The model was energy minimized using the CHARMM potential as implemented in QUANTA. A review of the final model highlighting residues of particular interest is shown in Fig. 1.

#### Construction of the chimeric AUK12-20 antibody

Using PCR methods, the cDNAs coding for the mouse AUK12-20  $V_L$  and  $V_H$  regions were modified to have HindIII sites and Kozak sequences at the 5' sides, and BamHI sites and splice donor sequences at the 3' sides (Maeda *et al.*, 1991; Kettleborough *et al.*, 1991). The V regions were then linked to the genes encoding human kappa or human gamma-1 constant regions as present in the HEF expression vectors (Sato *et al.*, 1993) (Fig. 2).

#### Construction of the reshaped human AUK12-20 light chain

The gene coding for reshaped human AUK12-20  $V_L$  region was constructed by the PCR-based CDR-grafting method shown in Fig. 3A. A plasmid DNA, pUC-RV1-PM1a-4, which was based on human RE1 (Sato *et al.*, 1993), was used as the template DNA. The final PCR product was digested with BamHI and HindIII and subcloned into a pUC19 vector. After DNA sequencing, the BamHI-HindIII DNA fragment encoding the reshaped human AUK12-20  $V_L$  region was excised from the plasmid DNA and inserted into the HEF expression vector yielding RV1-1220a.

#### Construction of the HSG-I-based reshaped human AUK12-20 heavy chains

The first set of reshaped human AUK12-20  $V_H$  regions was designed based on the consensus amino acid sequence for human  $V_H$  regions belonging to subgroup I (HSG-I). Version "a" was constructed by the CDR-grafting method described above. A plasmid DNA, pUC-RVh-425a, which has FRs based on HSG-I (Kettleborough *et al.*, 1991), was used as the template DNA. The final PCR product was digested with HindIII and BamHI and subcloned into a pUC19 vector yielding pUC-RVh-1220a. Version "b" was constructed using a PCR-based mutagenesis protocol similar to that shown in Fig. 3A. PCR primers introducing mutations of Arg66 to Lys66 and of Leu71 to Val71 were designed and used in the first PCR with the external primers (Fig. 3A). A plasmid DNA, pUC-RVh-1220a, was used as the template DNA. Two first PCR products were purified and then subjected to the second PCR. The final PCR product was digested with HindIII and BamHI and then subcloned into a pUC19 vector yielding pUC-RVh-1220b. The remaining versions "c" and "d" were constructed with appropriate mutagenic PCR primers and template DNAs. After DNA sequencing, the BamHI-HindIII DNA fragments encoding versions "a", "b", "c" and "d" were excised from the plasmid DNAs and then subcloned into the HEF expression vector yielding RVh-1220a, RVh-1220b, RVh-1220c, and RVh-1220d, respectively.

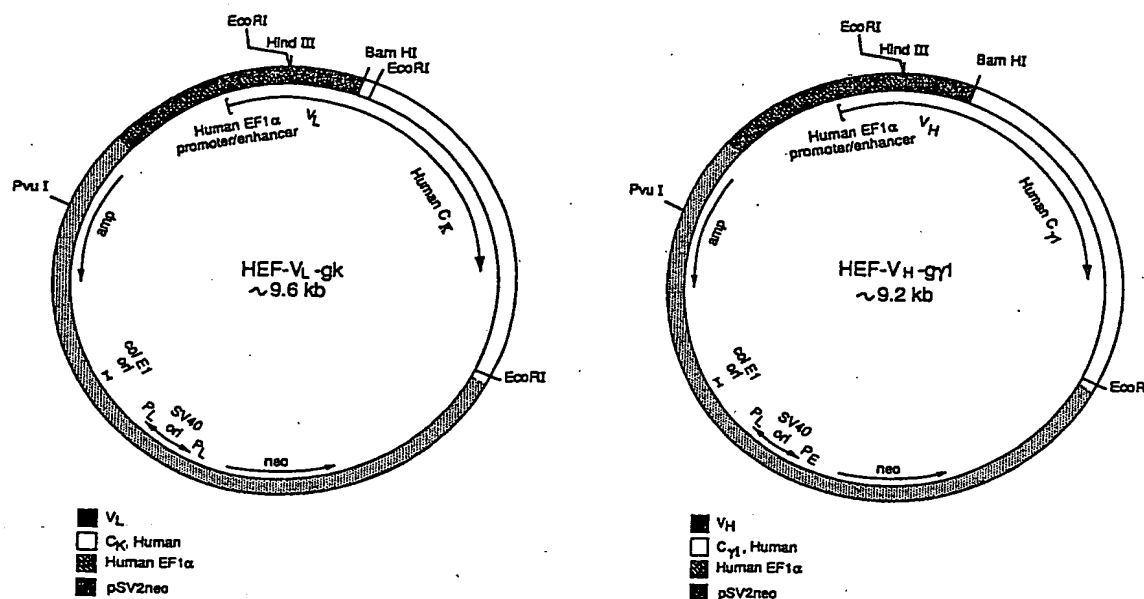


Fig. 2. Structures of the HEF expression vectors in which human elongation factor 1- $\alpha$  (EF1- $\alpha$ ) promoter-enhancer region is used. In these light and heavy chain expression vectors, V<sub>L</sub> and V<sub>H</sub> regions are linked to human kappa and gamma-1 constant regions, respectively.

#### Construction of the HAX-based reshaped human AUK12-20 heavy chains

The second set was designed based on the amino acid sequence of the human HAX V<sub>H</sub> region (Dersimonian *et al.*, 1987). The DNA coding for version "a" was designed based on the codon-usage observed in antibodies (Kabat *et al.*, 1991). After dividing the DNA sequence into six overlapping oligonucleotides, the oligonucleotides were analysed by computer for possible secondary structures that might interfere with assembly. The oligonucleotides were 90–94bp in length and had overlapping regions of 21bp. Two external PCR primers were also designed. As shown in Fig. 3B, the six oligonucleotides were assembled and a full-length DNA was amplified. The final PCR product was digested with BamHI and HindIII and subcloned into a pUC19 vector yielding pUC-sle1220Ha. The remaining versions "b", "c" and "d" were constructed with appropriate mutagenic PCR primers and template DNAs as described above. After DNA sequencing, the BamHI–HindIII DNA fragments encoding versions "a", "b", "c" and "d" were excised from the plasmid DNAs and subcloned into the HEF expression vector yielding sle1220Ha, sle1220Hb, sle1220Hc and sle1220Hd, respectively.

#### Expression and purification of antibodies

The light and heavy chain expression vectors were co-transfected into COS cells by electroporation. Equal amounts of each plasmid DNA (10  $\mu$ g) were added to 0.8 ml of cells suspended in PBS at  $1 \times 10^7$  ml<sup>-1</sup>. A pulse was delivered at 1.9 kV, 25  $\mu$ F capacitance using the Gene Pulsar apparatus (BioRad). After a 10 min recovery period at room temperature, the electroporated cells were added to 20 ml of DMEM containing 10% gamma globulin-free fetal calf serum (GIBCO). After

72 hr incubation, the medium was collected, centrifuged to remove cellular debris, and applied to a Protein A agarose column (Affi-Gel Protein A MAPSII kit, BioRad) equilibrated with binding buffer. After washing with binding buffer, antibodies were eluted with five bed volumes of the elution buffer. The elute was concentrated and the buffer changed to PBS using a microconcentrator (Centricon 100, Amicon).

#### Enzyme-linked immunoabsorbance assay

ELISA plates for the antigen-binding assay were prepared as follows. Ninety-six well-plates were coated with mouse MT18 antibody to the human IL-6R (Hirata *et al.*, 1989) which recognizes a different epitope from that of mouse AUK12-20 antibody. Following blocking, soluble recombinant human IL-6 receptor (SR344) (Yasukawa *et al.*, 1990) was added. After washing, samples of AUK12-20 antibodies were serially diluted and added to each well. Alkaline phosphatase-conjugated goat anti-human IgG was then added. After incubation and washing, substrate solution was added and the optical density at 405 nm was measured.

For the competition assay, ELISA plates were prepared as described above. The samples were serially diluted and added to the wells together with biotinylated human IL-6. Alkaline phosphatase-conjugated streptavidine was then added. The optical density at 405 nm was measured as described before.

#### Cell lines and growth inhibition assay

Human multiple myeloma cell lines, MMS1 (Okuno *et al.*, 1990) and ILKM3 (Shimizu *et al.*, 1989), were maintained in RPMI1640 supplemented with 20 or 10% FCS (GIBCO), respectively, in the presence of 2 ng/ml of recombinant human IL-6. A human T lymphoma cell

line, KT3 (Shimizu *et al.*, 1988) was also maintained in the same conditions described for ILKM3. In the growth inhibition-assay, 25 pg/ml of IL-6 was used for KT3 ( $1 \times 10^3$  cells) and 250 pg/ml of IL-6 was used for ILKM3 ( $5 \times 10^3$  cells) and MMS1 ( $4 \times 10^4$  cells). The cells were cultured for 5 days in 96 well plates in the presence of test samples. After pulse labeling with  $^3\text{H}$ -TdR for 6 hr, cells were harvested and transferred to a liquid scintillation counter.

## RESULTS

### Characterization of the mouse AUK12-20 variable regions

The mouse AUK12-20  $V_L$  region belongs to subgroup III of mouse kappa  $V_L$  regions and uses Jk2. The mouse AUK12-20  $V_H$  region belongs to subgroup IIA of mouse  $V_H$  regions and used J<sub>H</sub>3 (Kabat *et al.*, 1991). The mouse AUK12-20  $V_L$  and  $V_H$  regions were joined to human kappa and gamma-1 constant regions, respectively, to create a chimeric AUK12-20 antibody. This chimeric antibody bound well to human IL-6R thus indicating in a functional assay that the correct mouse AUK12-20  $V_L$  and  $V_H$  regions had been cloned (Fig. 5). In order to assist in the design of the reshaped human

AUK12-20 variable regions, a structure model of the mouse AUK12-20 variable regions was built (Fig. 1).

### Design of the reshaped human AUK12-20 variable regions

The mouse AUK12-20  $V_L$  region showed almost equal homology with the consensus sequences of all four subgroups of human kappa  $V_L$  regions (64.0–67.6% identity). Although the  $V_L$  region from human LEN antibody (Schneider and Hilschmann, 1975), a member of HSG-IV, showed the highest homology (68.8% identity), the  $V_L$  region from human REI (Palm and Hilschmann, 1975), a member of HSG-I, was chosen for the design of the reshaped human AUK12-20  $V_L$  region. The  $V_L$  region of REI also showed good homology (62.0% identity) and this human kappa light chain variable region has been used successfully as the basis of many reshaped human light chain variable regions. Thus reshaped human AUK12-20  $V_L$  region was designed based on the FRs from human REI (Fig. 4A). Five alterations were made in the human FRs at positions 39, 71, 104, 105 and 107 as compared to the original amino acid sequence of human REI. The three changes in FR4 (positions 104, 105 and 107) were based on a more typical J region from another human kappa light chain

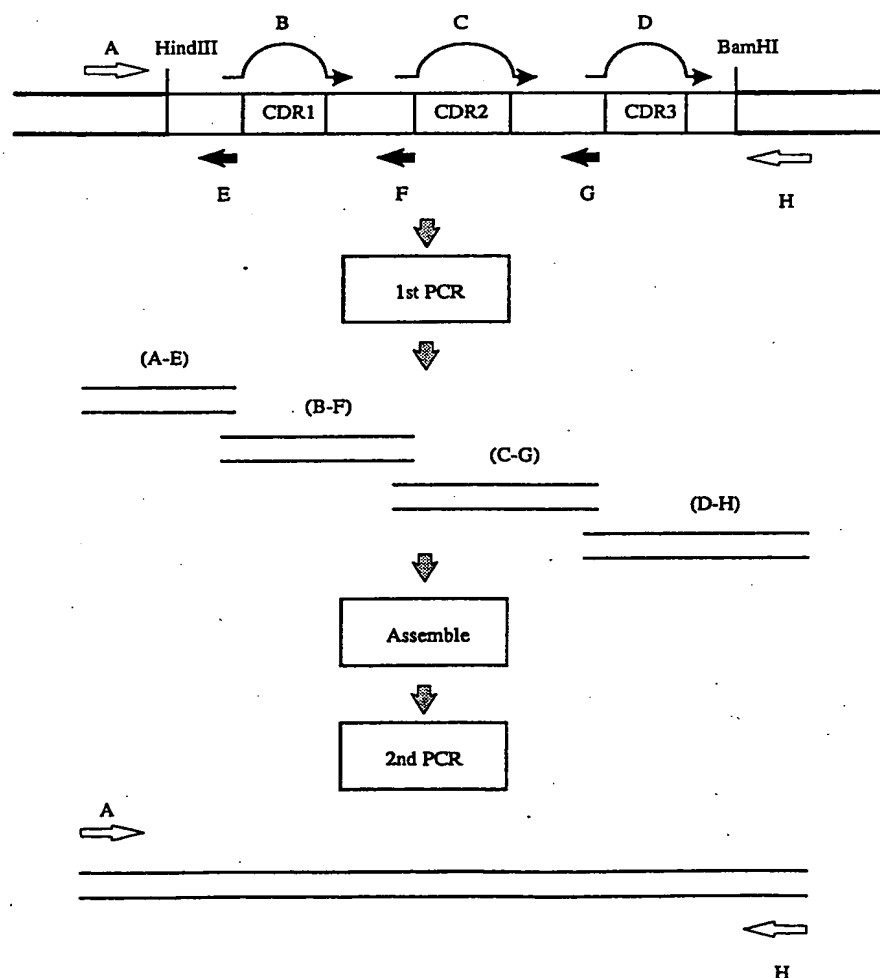


Fig. 3.(A).

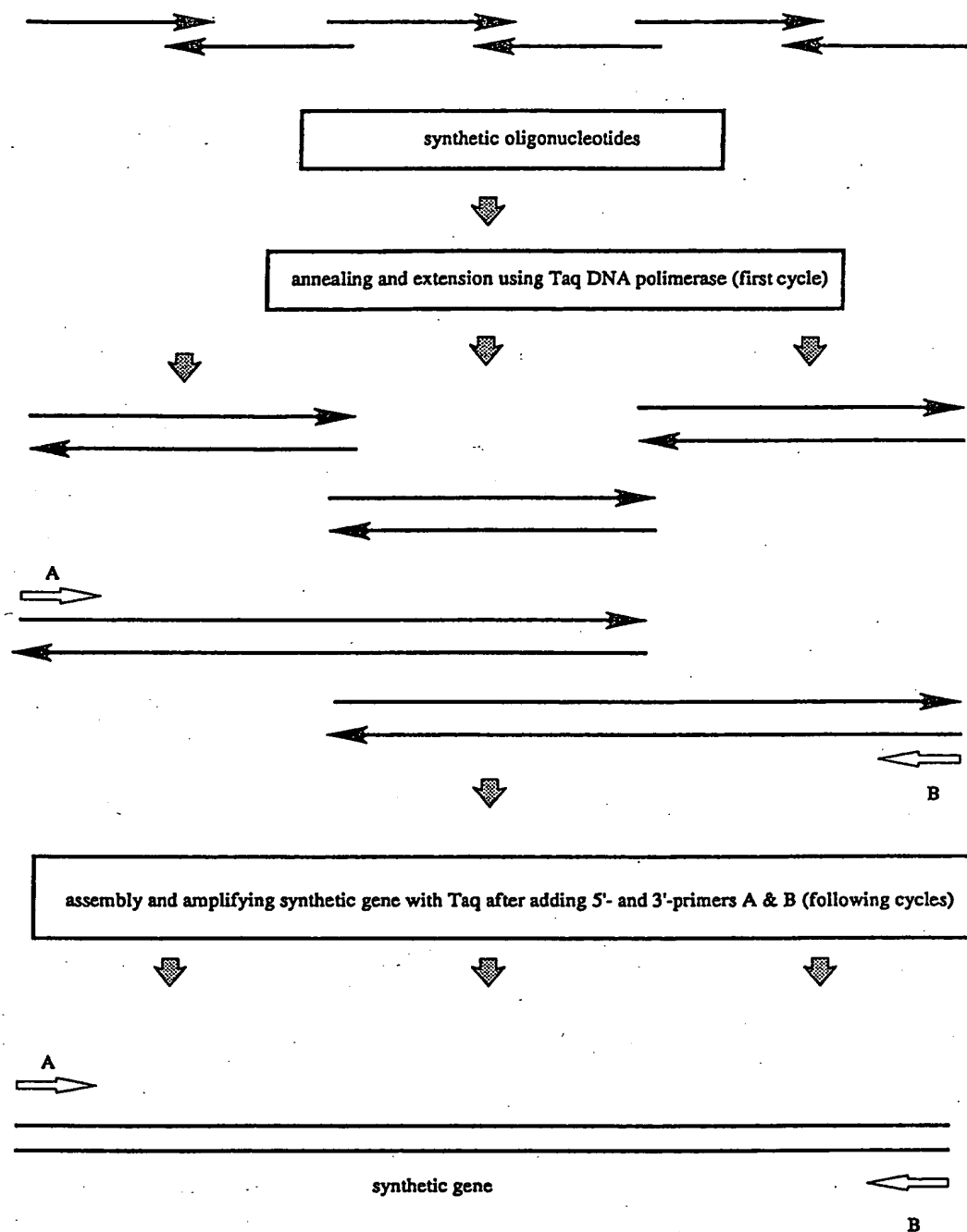


Fig. 3(B).

Fig. 3. Two PCR-based CDR-grafting methods. (A) Strategy used for the construction of version "a" of the REI-based reshaped human AUK12-20  $V_L$  region and version "a" of the HSG-I-based reshaped human AUK12-20  $V_H$  region. Eight PCR primers were designed. The external primers A and H hybridize to DNA sequences in the vector. The CDR-grafting primers B, C and D have the DNA sequences coding for CDR1, CDR2 and CDR3, respectively, of mouse AUK12-20 variable regions. The complementary primers E, F and G consist of 15–20 bases which are the complementary DNA sequences on the 5'-side of primers B, C and D, respectively. In the first PCR step, four reactions A–E, B–F, C–G and D–H were carried out with a template DNA. In the second PCR step, the four PCR products from the first PCR step were assembled by their own complementarity (15–20 bases). External primers (A and H) were then added to the reaction and the full-length DNA product was amplified. The PCR products were digested with HindIII and BamHI and subcloned into a pUC19 vector. (B) Strategy used for the construction of version "a" of the HAX-based reshaped human AUK12-20  $V_H$  region. The DNA sequence coding for sle1220Ha was designed and divided into six overlapping oligonucleotides (90–94 bp). Three of the oligonucleotides have sense DNA sequence and the other three have anti-sense DNA sequence. Two external primers, A and B, were also designed. The six oligonucleotides were assembled and the full-length DNA was amplified with the external primers. The PCR product was digested with BamHI and HindIII and subcloned into a pUC19 vector.

Fig. 4. Design of reshaped human AUK12-20 V<sub>L</sub> (A) and V<sub>H</sub> (B) regions. Amino acid are numbered according to Kabat *et al.* (1991). The FRs for REI are the authentic human REI sequences as published by Palm and Hilschmann (1975). The FRs of the consensus sequence for HSG-1 are according to Kabat *et al.* (1991). At positions with an X, there was no single preferred amino acid in the consensus sequence. The FRs for HAX were based on a human hybridoma, isolate 21/28, producing an anti-DNA autoantibody from a systemic lupus erythematosus patient (Dersimonian *et al.*, 1987).

FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4			
1	2	4	5	6	7	8	9	10	
1	2	3	4	5	6	7	8	9	10

[illegible]

	FR1	FR2	CDR1	CDR2	FR3	CDR3	FR4				
	1	2	3	4	5	6	7	8	9	10	11
123456789012345678901234567890				67890123456789	01233456789012345	67890123456789012345	67890123456789012345	67890123456789012345	6789012345678901234	56789012	34567890123
AUK12-20	EQIQQQSGPELAKPGASVKLSCKASGYST			NVQSHGKSLWIG	YIDPFNGGTSYNQKYG	KATVTWKSSTAYMELSLTSEDSAVYYCAR	YIDPFNGGTSYNQKYG	YIDPFNGGTSYNQKYG	YIDPFNGGTSYNQKYG	CGN-RFAY	WGQGTLLVTSVA
HSOI	QVQLVQSGALEVKKPGASVKVSCKASGYTS			WVRQAPGQGLEWVG			RVETTDKSSKNTAYMELSLRSEDYAVYYCAR	RVETTDKSSKNTAYMELSLRSEDYAVYYCAR	RVETTDKSSKNTAYMELSLRSEDYAVYYCAR	CGN-RFAY	WGQGTLLVTSVS
RV <sub>A</sub> 1220a	QVQLVQSGALEVKKPGASVKVSCKASGYST			WVRQAPGQGLEWVG	YIDPFNGGTSYNQKYG		RVETMTLDTSTNTAYMELSLRSEDYAVYYCAR	RVETMTLDTSTNTAYMELSLRSEDYAVYYCAR	RVETMTLDTSTNTAYMELSLRSEDYAVYYCAR	CGN-RFAY	WGQGTLLVTSVS
RV <sub>A</sub> 1220b			-8-T				K-----V-----	K-----V-----	K-----V-----	---	---
RV <sub>A</sub> 1220c			-8-T				K-----V-----	K-----V-----	K-----V-----	---	---
RV <sub>A</sub> 1220d			-8-T				K-----V-----	K-----V-----	K-----V-----	---	---

(2) Based on the FRs in human HLA											
	FR1	2	3	CDR1	FR2	5	6	7	8	9	11
123456789012345678901234567890	12345	678901234567890	1234567890	12345	67890123456789	0123456789012345	67890123456789012345	67890123456789012345	67890123456789012345	56789012	34567890123
AVK12-20	EQIQQQSGPELMPGASVKISCKASGYFT			SIYIH	WVKSHGKSLIEWIG	YIDPFNGCTSYNQKPKG			KATLTVDKSSPAYMELSSLTSEDSAVTYCAR	CGN-RFAY	WGQGTLTVTSSA
HA	QVQLVQSGAEVKKPKQASVYVSKASGYFT			SIYIH	WVRQAPGQRLRWNG	YIDPFNGCTSYNQKPKG			RVTITRDTSASPAYMELSSLRSSEDAVTYCAR	CGN-RFAY	WGQGTLTVTSS
sle1220Ha	QVQLVQSGAEVKKPKQASVYVSKASGYFT			SIYIH	WVRQAPGQRLRWNG	YIDPFNGCTSYNQKPKG			RVTITVDTSASPAYMELSSLRSSEDAVTYCAR	CGN-RFAY	WGQGTLTVTSS
sle1220Hb	-----S-----			-----S-----	-----I-----			-----V-----	-----	-----	-----
sle1220Hc	-----S-----			-----S-----	-----I-----			X-----V-----	-----	-----	-----
sle1220Hd	-----S-----			-----S-----	-----I-----			X-----V-----	-----	-----	-----



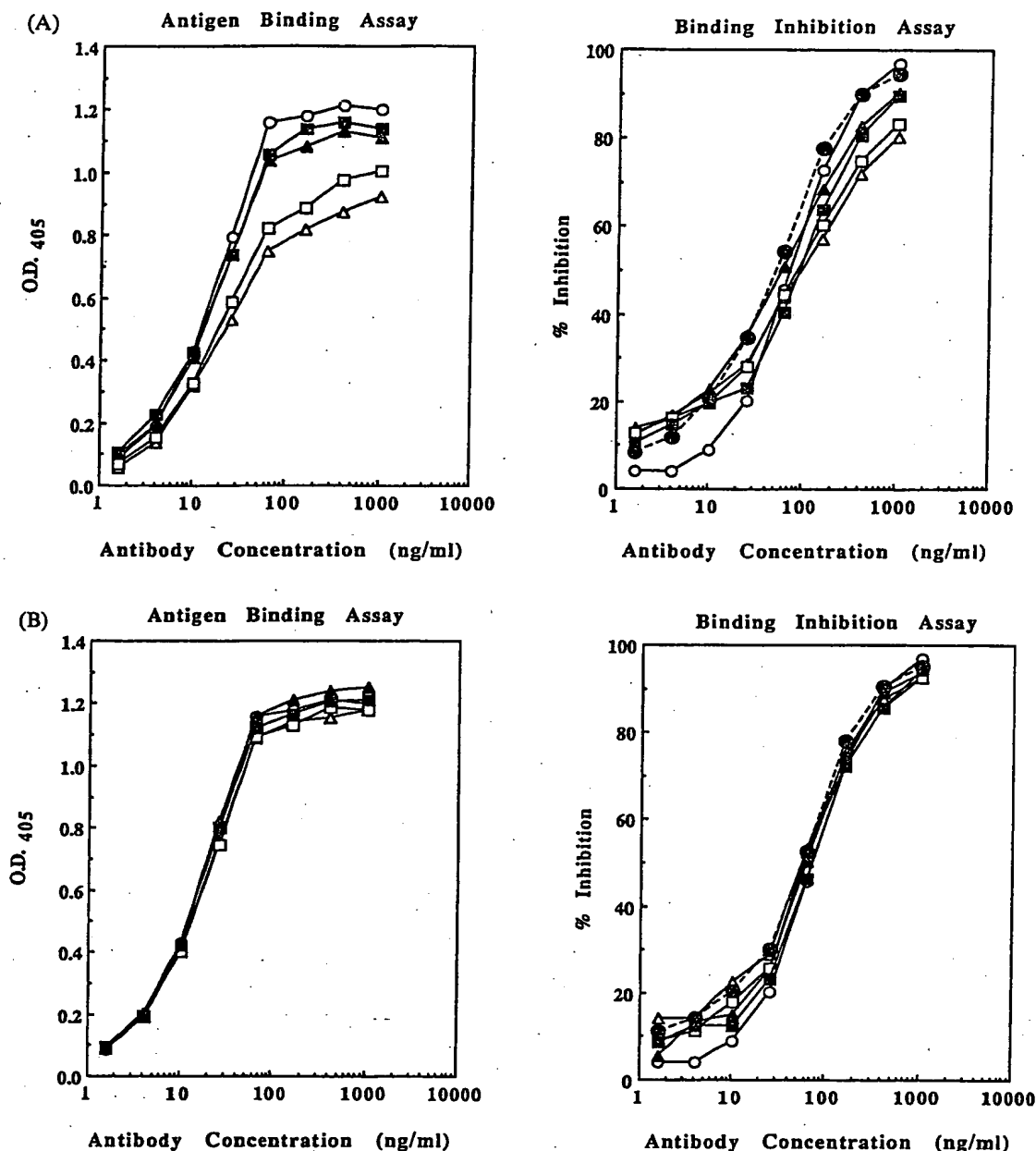


Fig. 5. Analysis of the reshaped human AUK12-20 antibodies in combinations of the REI-based reshaped light chain, RV1220a, and eight versions of reshaped heavy chains. (A) Antigen-binding assay (left) and binding-inhibition assay (right) of the HSG-I-based reshaped heavy chains, RVh1220a ( $\Delta$ ), RVh1220b ( $\blacktriangle$ ), RVh1220c ( $\square$ ) and RVh1220d ( $\blacksquare$ ). (B) Antigen-binding assay (left) and binding-inhibition assay (right) of the HAX-based reshaped heavy chains, sle1220Ha ( $\Delta$ ), sle1220Hb ( $\blacktriangle$ ), sle1220Hc ( $\square$ ) and sle1220Hd ( $\blacksquare$ ). In these assays, chimeric ( $\circ$ ) and mouse AUK12-20 ( $\bullet$ ) antibodies were used as positive controls.

and, therefore, do not constitute a deviation from human sequences (Riechmann *et al.*, 1988). The amino acid residues at positions 39 and 71 were changed to the amino acids present in the mouse AUK12-20 sequence. Almost all of the  $V_L$  regions of human kappa light chains have a lysine at position 39 suggesting that this alteration is not a deviation from human sequences. Position 71 is particularly important because the amino acid at this position is part of the canonical structure for L1 and is important in the packing of the L1 loop (Chothia *et al.*,

1989; Foote and Winter, 1992). This one version of reshaped human AUK12-20  $V_L$  region was joined to a human kappa constant region and co-expressed with chimeric AUK12-20 heavy chain. It gave binding to antigen that was equivalent to that of chimeric AUK12-20 antibody.

In the design of the reshaped human AUK12-20  $V_H$  regions, two methods for selecting human FRs were tried. The first method was based on selecting human FRs from the most homologous consensus sequence for

the three subgroups of human  $V_H$  regions. The FRs from mouse AUK12-20  $V_H$  region were most similar (59% identity) to the consensus sequence for HSG-I. Thus the first set of four versions of reshaped human AUK12-20  $V_H$  regions was designed based on the FRs from the consensus sequence for HSG-I (Fig. 4B). The template DNA for constructing these four versions was version "a" of reshaped human 425  $V_H$  region which was also designed based on the FRs of HSG-I (Kettleborough *et al.*, 1991). Five amino acid residues in the FRs of HSG-I (positions 28, 30, 48, 66 and 71) were identified as having a possible adverse effect on antigen binding (Fig. 1). Residues 28 and 30 in FR1 are part of the structural loop that forms the H1 loop and, therefore, Ser28 and Thr30 are likely to be directly involved in antigen-binding site (Chothia *et al.*, 1989; Chothia and Lesk, 1987). Residue 48 in FR2 is buried under the H2 loop and, therefore, may influence the conformation of this loop (Kettleborough *et al.*, 1991). Residue 66 in FR3 is immediately adjacent to H2 and could influence the conformation of the H2 loop. Residue 71 in FR3 is part of the canonical structure for H2 and is important in the packing of the H2 loop (Chothia *et al.*, 1989; Tramontano *et al.*, 1990). In the model, the valine at position 71 allows the proline at position 52A in the H2 loop to pack into a small cavity (Fig. 1).

The second method for designing the reshaped human AUK12-20  $V_H$  regions was based on selecting the human FRs from the most homologous human  $V_H$  region. Human HAX  $V_H$  region was most similar to mouse AUK12-20  $V_H$  region (66% identity) and also had CDRs of sizes similar to those in mouse AUK12-20  $V_H$  region. Thus the second set of four versions of reshaped human AUK12-20  $V_H$  region was designed based on the FRs from human HAX  $V_H$  region (Fig. 4). Four amino acid residues in the FRs of HAX (positions 30, 48, 66 and 71) were identified as possibly influencing antigen binding. The importance of these four positions was discussed previously.

#### *Analysis of reshaped human AUK12-20 antibodies*

Each reshaped human AUK12-20 heavy chain was co-expressed with the reshaped human AUK12-20 light chain (RV1220a). The resulting antibodies were analysed by ELISA for binding to human IL-6R. Of the four versions of reshaped human AUK12-20  $V_H$  regions based on HSG-I, versions "b" and "d" (RVh1220b and RVh1220d) bound to antigen approximately 90% as well as chimeric AUK12-20 antibody. Versions "a" and "c" (RVh1220a and RVh1220c) bound only approximately 60–70% as well as chimeric antibody (Fig. 5A). Of the four versions of reshaped human AUK12-20  $V_H$  regions based on HAX, all four versions (sle1220Ha, sle1220Hb, sle1220Hc and sle1220Hd) bound to antigen as well as chimeric AUK12-20 antibody (Fig. 5B).

Next, reshaped human AUK12-20 antibodies were tested for the ability of IL-6 to inhibit their binding to IL-6R. In this assay, the reshaped human AUK12-20 antibodies could be compared directly to both the mouse and chimeric AUK12-20 antibodies. The results from

these binding inhibition assays confirmed the results obtained from the antigen binding assays (Fig. 5).

Based on their binding to antigen and the number of alterations in the human FRs, two reshaped human AUK12-20 antibodies (RV1220a + RVh1220b and RV1220a + sle1220Ha) were selected for further analysis. Both of these reshaped human AUK12-20 antibodies inhibited the IL-6-dependent growth of several tumor cell lines (Fig. 6). The  $IC_{50}$ s of the reshaped human AUK12-20 antibodies were almost equal to those of mouse and chimeric AUK12-20 antibodies (approximately 40 ng/ml for KT3 cells, 500 ng/ml for ILKM3 cells and 800 ng/ml for MMS1 cells) indicating that these reshaped human AUK12-20 antibodies are functionally equivalent to the original mouse AUK12-20 antibody in these assays. These reshaped human AUK12-20 antibodies, therefore, could be efficacious in human patients with IL-6 related disorders.

### DISCUSSION

This report describes the design and construction of reshaped human AUK12-20 antibodies specific for human IL-6R. Several alterations in the human FRs are usually required to recreate a good functional antigen-binding site in a reshaped human antibody. The number of changes in the human FRs, however, should be minimized to avoid introducing immunogenic in human patients. Very minor alterations were required for the REI-based reshaped human AUK12-20  $V_L$  region. This reshaped human AUK12-20 light chain was equivalent to the chimeric AUK12-20 light chain in antigen binding assays.

We compared two approaches for designing the reshaped human AUK12-20  $V_H$  regions. We designed the  $V_H$  regions based on the most homologous consensus sequence and on the most homologous individual human antibody. Both approaches have been used successfully but have not been applied to the same antibody and carefully compared. From the set of reshaped human  $V_H$  regions based on a consensus sequence (HSG-I), the best version was "b" (RVh1220b). It showed approximately 90% of the activity of chimeric antibody in antigen binding assays and binding inhibition assays. In addition, there were only four alterations in the human FRs at positions 28, 30, 66 and 71.

From the set of reshaped human  $V_H$  regions based on an individual human antibody (HAX), it was difficult to select the best version. All four versions were identical to chimeric antibody in antigen binding assays. Version "a" was selected as the best version from this set of four reshaped human AUK12-20  $V_H$  regions because it had the fewest alterations in the human FRs. There were only two alterations at positions 28 and 71.

Comparing the best versions of reshaped human AUK12-20  $V_H$  regions from the two methods of design shows that they differ at only six positions (44, 48, 66, 69, 75 and 76). The difference at position 44 is probably the most significant with a change from a glycine in RVh1220b to an arginine in sle1220Ha.

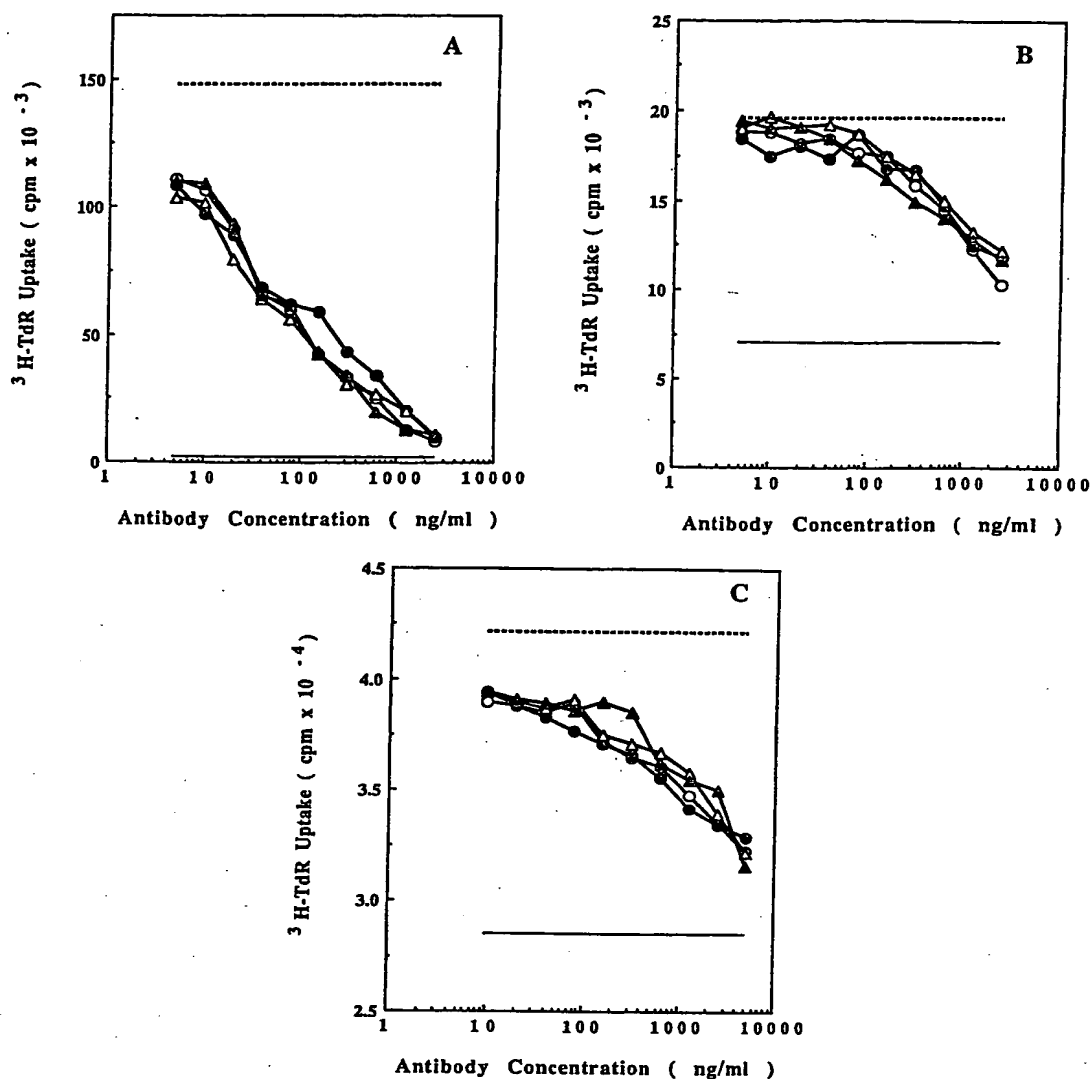


Fig. 6. Comparison of the effect of AUK12-20 antibodies on IL-6-dependent growth of three tumor cell lines, (A) KT3, (B) ILKM3 and (C) MMS1. Reshaped antibodies consist of RV11220a and RVh1220b (●), and sle1220Ha (○). Chimeric (△) and mouse AUK12-20 (▲) antibodies were used as positive controls. Here (----) and (—) show  $^3\text{H}$  thymidine uptake with or without IL-6, respectively.

We have shown that both approaches to designing reshaped human variable regions can lead to successful humanized antibodies. Designing based on a consensus sequence may be preferable to designing based on the sequence from an individual human antibody in that the consensus sequence will filter out any unusual, and possibly more immunogenic, sequences peculiar to an individual human antibody. On the other hand, the "averaging" of numerous human sequences to create a consensus sequence may create an artificial unnatural sequence that could be immunogenic. Our results designing and testing eight versions of reshaped human AUK12-20  $V_H$  region indicate that designing based on an individual human antibody  $V_H$  region is best. This method gave us reshaped human antibodies with the best binding to antigen and with the least requirement for alterations in the human FRs. We

recommend that reshaped human variable region be designed based on the most homologous human variable regions and that the resulting design be compared to the consensus sequence for that subgroup of human variable regions to identify any possible highly irregular sequences.

Most importantly, we have designed and constructed reshaped human AUK12-20 antibodies that inhibit the growth of IL-6-dependent tumor cell line as well as the original mouse AUK12-20 antibody. These results suggest that the reshaped human AUK12-20 antibodies will be efficacious in treating human patients with IL-6-dependent tumors.

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